JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2007, p. 2392–2397 Vol. 45, No. 8
0095-1137/07/$08.00+0 doi:10.1128/JCM.00556-07

Rapid Identification of Viridans Streptococci by Mass Spectrometric Discrimination▽

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Received 13 March 2007/Returned for modification 20 April 2007/Accepted 29 May 2007

Viridans streptococci (VS) are responsible for several systemic diseases, such as endocarditis, abscesses, and septicemia. Unfortunately, species identification by conventional methods seems to be more difficult than species identification of other groups of bacteria. The aim of the present study was to evaluate the use of cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) for the rapid identification of 10 different species of VS. A total of 99 VS clinical isolates, 10 reference strains, and 20 strains from our in-house culture collection were analyzed by MALDI-TOF-MS. To evaluate the mass-spectrometric discrimination results, all strains were identified in parallel by phenotypic and genotypic methods. MALDI-TOF-MS identified 71 isolates as the mitis group, 23 as the anginosus group, and 5 as *Streptococcus salivarius*. Comparison of the species identification results obtained by the MALDI-TOF-MS analyses and with the phenotypic/genotypic identification systems showed 100% consistency at the species level. Thus, MALDI-TOF-MS seems to be a rapid and reliable method for the identification of species of VS from clinical samples.

Established methods for bacterial identification in clinical microbiology are often time-consuming and do not always lead to a reliable differentiation of closely related species. Hence, there is an increasing need for alternative procedures that allow the rapid and reliable identification of microorganisms. Bacterial identification by matrix-assisted laser desorption ionization–time of light mass spectrometry (MALDI-TOF-MS) holds the potential to serve this need.

Viridans streptococci (VS) are commensal bacteria of the human oral cavity and the respiratory, gastrointestinal, and genitourinary tracts. On the other hand, they are responsible for several systemic diseases, including subacute infective endocarditis, septicemia, meningitis, and pyogenic infections (1, 9, 17). The heterogeneous group of VS currently includes more than 30 species. They form five major groups, namely, the mutans, salivarius, anginosus, mitis, and bovis groups (16). The accurate species-level identification of isolates from relevant clinical specimens, like blood and abscess material, is important in understanding the pathogenic mechanisms of the particular species. Unfortunately, species identification seems to be more difficult for VS than for other groups of bacteria, possibly because VS are competent bacteria and, thus, may readily take up DNA from the environment.

Phenotypic test systems do not always allow the accurate identification of some species in this heterogeneous group of bacteria (3, 10). Several molecular methods for the identification of VS to the species level have been developed. The targets of the molecular methods are, e.g., the 16S RNA gene, the 16S-23S rRNA gene intergenic spacer region (7), the D-alanine–D-alanine ligase gene (11), hyaluronate lyase genes (27), and the glucosyltransferase gene (14). Species of the mitis group are especially difficult to differentiate by these methods (C. Friedrichs et al., submitted for publication). This may be because the nucleotide sequences of the 16S rRNA genes of *Streptococcus mitis*, *S. oralis*, *S. pseudopneumoniae*, and *S. pneumoniae* match by more than 99% (16).

Several gram-negative bacteria, gram-positive bacteria, and *Mycobacterium* species have been analyzed by MALDI-TOF-MS (8, 21, 29). Beta-hemolytic streptococci could be arranged into group A, C, and G streptococci by MALDI-TOF-MS analysis (18).

The differentiation of oral streptococci at the species level and the mutans streptococci at the subspecies level was achieved by MALDI-TOF-MS (25). The aim of the present study was to evaluate the use of MALDI-TOF-MS for the rapid identification of 10 different species of VS isolated from relevant clinical samples.

MATERIALS AND METHODS

Chemicals. Protein calibration standard I and α-cyano-4-hydroxycinnamic acid were purchased from Bruker Daltonics, Bremen, Germany; trifluoroacetic acid (TFA) was from Merck, Darmstadt, Germany; and acetonitrile was from Sigma, Taufkirchen, Germany. Deionized water was used in all experiments. Columbia agar base and the other media used were purchased from Oxoid, Basingstoke, United Kingdom.

Bacterial strains, culture, and phenotypic biochemical and genotypic identification. A total of 99 consecutive clinical isolates of VS were recovered from blood cultures ($n = 25$), aspirates of peritonsillar abscesses ($n = 21$), several other abscesses ($n = 18$), wound swabs ($n = 29$), catheter tips ($n = 5$), and cerebrospinal fluid ($n = 1$). All isolates were subcultured at 37°C in 5% CO_2 on 5% sheep blood agar and chocolate agar. The strains were all preliminarily identified as VS on the basis of alpha-hemolysis, colony characteristics, a positive Gram stain reaction, coccus morphology in chains, and a negative catalase test result. Pneumococci and enterococci were eliminated by routine biochemical assays, like the optochin test and bile solubility, pyrrolidonylarylamidase, and oxacillin testing. The isolates were biochemically identified with the Rapid ID 32 STREP system (bioMérieux, Lyon, France); and genotypic identifications were

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▽ Published ahead of print on 6 June 2007.

performed by previously described species-specific PCRs for *S. oralis* (14); *S. gordonii*, *S. mitis*, *S. mutans*, *S. salivarius*, and *S. sanguinis* (11); *S. anginosus*, *S. constellatus*, and *S. intermedius* (27); and *S. parasanguinis* (Friedrichs et al., submitted) or, in some cases, by sequence analysis of the 16S rRNA gene (5, 7).

Standard samples. The following reference strains were used in this study for MALDI-TOF-MS analyses: *S. intermedius* DSM 20573, *S. mutans* DSM 20523, *S. oralis* DSM 20627, *S. parasanguinis* DSM 6778, *S. salivarius* DSM 20560, *S. sanguinis* DSM 20567, *S. mitis* DSM 12643, *S. constellatus* subsp. *constellatus* DSM 20575, *S. anginosus* DSM 20563, and *S. gordonii* DSM 6777 (DSMZ, Braunschweig, Germany). In addition to these strains, we selected from our in-house culture collection two additional strains of each species on the basis of a coherent previous identification by phenotypic and genotypic methods.

Sample preparation for MALDI-TOF-MS. Individual colonies of the respective bacterial species were recultured in brain heart infusion overnight. One milliliter of bacterial suspension was centrifuged at 7,500 × *g* for 15 min. The sediment was washed twice with deionized water and then dissolved in 50 μl 80% TFA. After 10 min incubation at room temperature 150 μl deionized water and 200 μl acetonitrile were added. The samples were stored at −20°C. After the samples were thawed, they were centrifuged at 13,000 rpm for 2 min. The supernatant was transferred into a 1.5-ml Eppendorf tube and dried in a vacuum centrifuge. The pellet was dissolved in 20 μl 2.5% TFA–50% acetonitrile. One microliter was pipetted onto the stainless steel MALDI target plate. After the spots dried, they were overlaid with 1.0 μl of matrix (α-cyano-4-hydroxycinnamic acid as a saturated solution in 2.5% TFA–50% acetonitrile). The matrix/sample spots were crystallized by air drying.

MALDI-TOF-MS parameters. All mass spectra were acquired with an Autoflex (Bruker Daltonics, Bremen, Germany) MALDI-TOF mass spectrometer with a nitrogen laser (337 nm) operated in the positive linear mode (delay, 150 ns; voltage, 20 kV; mass range, 2 to 50 kDa) under the control of Flexcontrol software (version 2.4; Bruker Daltonics). Each spectrum was obtained by averaging 500 laser shots acquired in the automatic mode at the minimum laser power necessary for ionization of the samples. The spectra were externally calibrated by using the standard calibration mixture, Protein Calibration Standard I, supplied by Bruker Daltonics. The data files were transferred to the Flexanalysis software (version 2.4; Bruker Daltonics) for automated peak extraction.

MALDI-TOF-MS statistical analysis. With the Flexanalysis software, 40 peaks were automatically labeled in each spectrum according to their appearance above the background (threshold ratio, 1.5). Correct labeling was controlled manually. Peak lists containing masses and intensities were exported as ASCII files. Similarity analysis between peak lists was carried out by using a hierarchical clustering procedure performed with MatLab software (version 7.3; The MathWorks Inc., Natick, MA). To identify the corresponding peaks in the different spectra, a mass window was defined around each peak. The mass window considers the differences of masses assigned to identical peaks in different samples. A mass-dependent size of the mass window was chosen according to the following equation: mass window size = $\text{size}_{\text{abs}} + (\text{mass} \cdot \text{size}_{\text{rel}})$, where size_{abs} is the absolute mass-dependent size and size_{rel} is the relative mass-dependent size.

Typically, a size_{abs} of 1 Da and a size_{rel} of 1.001 were applied. Peaks originating from different spectra and occurring in the same window were assigned to one "cluster." If neighboring clusters overlapped, they were omitted from further calculations. The similarity between spectra was determined by pairwise comparison of the spectra by counting the number of clusters to which the two spectra contributed. By this procedure, a symmetric matrix of pairwise similarities (peak mass-based similarity matrix) was formed. In addition, a similarity matrix ($\sigma_{i,j}$), which considers peak masses and differences in the peak intensities, was calculated according to the following equation:

$$\sigma_{i,j} = \sum_k \left[1 - \left(\frac{w_i^k - w_j^k}{w_i^k + w_j^k} \right)^2 \right]$$

The similarity of samples i and j was obtained by summation over all clusters k contributing to either sample i or sample j. w_i^k represents the intensity of peak i in spectrum k.

Distance matrices ($\delta_{i,j}$) were calculated from normalized similarity matrices according to the following equation:

$$\delta_{i,j} = 1 - \sigma_{i,j}$$

Dendrograms were calculated on the basis of the distance matrices by using a complete linkage function.

For classification of the spectra, the support vector machine (SVM) tool implemented in the Bioinformatic toolbox of MatLab (version 7.3; The MathWorks Inc.) was used. This software, which features an efficient two-class classification, enables the user to define a number of parameters and to select from a choice of built-in kernel functions, including a radial basis function and a polynomial kernel (of a given degree). The SVM algorithm (31) was trained with a set of spectra of bacteria of known identity. An error estimate of the class prediction was carried out by calculation of a 10-fold cross-validation error for the training group. For this purpose, the training set was first divided into 10 subsets of equal size. Sequentially, one subset was tested by using the classifier trained on the remaining nine subsets. Thus, each probe of the training set was predicted once. The cross-validation accuracy is the percentage of data which were correctly classified.

RESULTS AND DISCUSSION

The reference strains of VS investigated showed characteristic MALDI-TOF-MS spectra (typical examples are given in Fig. 1). The majority of peaks were obtained below a mass/charge (m/z) of 8,000. No peaks were observed above an m/z of 10,000. Already from a visual inspection, an overall gross similarity of all spectra, which reflects the common genus, can be recognized. However, distinct differences between the spectra of different species are also visible.

The spectra obtained for the reference strains and the culture collection strains were used for similarity analysis, as described in Materials and Methods. Figure 2 shows a dendrogram of the different VS calculated from a peak mass-based similarity matrix. The reproducibility of the method can be deduced from the similarity of spectra 1 and 2 of each species, which were obtained from two independent cultures of the same reference strain processed on different days, respectively. It can further be seen that the spectra of different species cluster separately and that the spectra of the culture collection strains cluster with those of the respective reference strains. From the tree structure it can further be deduced that the phenotypic relations between the VS analyzed closely resemble their genetic relationships. Generally, the spectra of genetically closely related species (e.g., in the anginosus group) are more similar to each other than to those of genetically more distant VS (e.g., *S. salivarius* and *S. mutans*).

The obtained results prompted us to create a MALDI-TOF-MS spectrum database (VS database) from the spectra of the reference strains and the culture collection strains cultured two to three times in parallel with the intention of using it as a basis for the identification of unknown VS isolates. As an example, Fig. 3 shows the results of the analysis of one clinical isolate (isolate VS 162). The MALDI-TOF-MS spectrum of this isolate was compared to the spectra in the VS database by similarity analysis. On the y axis the distance between the spectrum of VS 162 and the spectra of the reference strains is given. On the x axis the reference strains are arranged according to the increasing dissimilarities of their MALDI-TOF-MS spectra to the spectrum of VS 162. For clarity, only the results for the 20 most similar references are shown. It can be seen that all spectra for the *S. sanguinis* reference strains are more similar to the spectrum of VS 162 than to the spectrum of any other reference strain. Therefore, this isolate can unambiguously be identified as *S. sanguinis* on the basis of MALDI-TOF-MS analysis. All clinical isolates shown in Table 1, which were identified to the species level by conventional microbiological methods (with the API system and by species-specific PCR and 16S rRNA gene sequencing), had identical results by

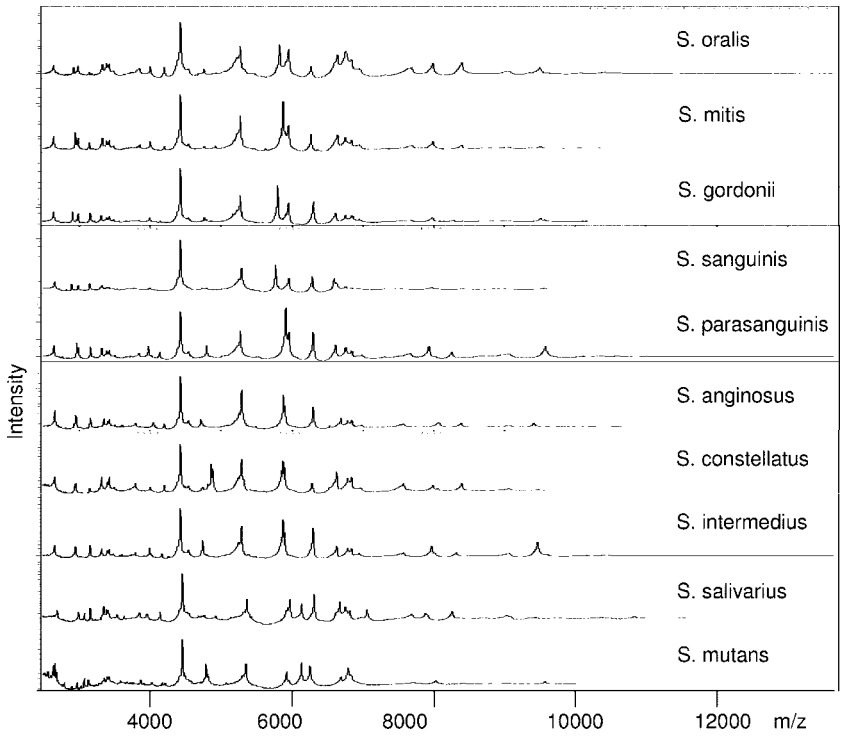

FIG. 1. MALDI-TOF mass spectra of different streptococcal reference strains: *S. oralis* DSM 20627, *S. mitis* DSM 12643, *S. gordonii* DSM 6777, *S. sanguinis* DSM 20567, *S. parasanguinis* DSM 6778, *S. anginosus* DSM 20563, *S. constellatus* DSM 20575, *S. intermedius* DSM 20573, *S. salivarius* DSM 20560, and *S. mutans* DSM 20523.

MALDI-TOF-MS similarity analysis performed with the spectra obtained from two independent cultures of each isolate.

The 23 samples which could be identified as either *S. mitis* or *S. oralis* by species-specific PCR and sequence analysis of the 16S RNA gene (Table 1, *S. oralis*/*S. mitis*) were also studied by similarity analysis of their MALDI-TOF-MS spectra by use of the VS database as a reference. In all but two cases the isolates could definitely be identified as either *S. oralis* (an example is

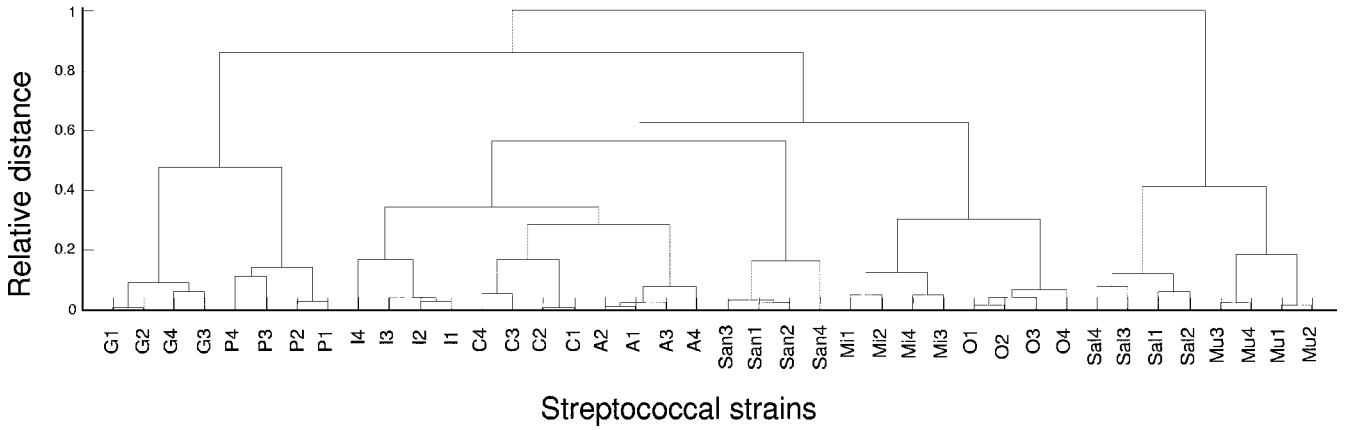

FIG. 2. Results of similarity analysis of reference strains and additional reference strains. The dendrogram was calculated from a peak mass-based similarity matrix. G1 and G2, *S. gordonii* DSM 6777, respectively; G3 and G4, two different culture collection strains of *S. gordonii*, respectively; P1 and P2, two colonies of *S. parasanguinis* DSM 6778, respectively; P3 and P4, two different culture collection strains of *S. parasanguinis*, respectively; I1 and I2, two colonies of *S. intermedius* DSM 20573, respectively; I3 and I4, two different culture collection strains of *S. intermedius*, respectively; C1 and C2, two colonies of *S. constellatus* DSM 20575, respectively; C3 and C4, two different culture collection strains of *S. constellatus*, respectively; A1 and A2, two colonies of *S. anginosus* DSM 20563, respectively; A3 and A4, two different culture collection strains of *S. anginosus*, respectively; San1 and San2, two colonies of *S. sanguinis* DSM 20567, respectively; San3 and San4, two different culture collection strains of *S. sanguinis*, respectively; Mi1 and Mi2, two colonies of *S. mitis* DSM 12643, respectively; Mi3 and Mi4, two different culture collection strains of *S. mitis*, respectively; O1 and O2, two colonies of *S. oralis* DSM 20627, respectively; O3 and O4, two different culture collection strains of *S. oralis*, respectively; Sal1 and Sal2, two colonies of *S. salivarius* DSM 20560, respectively; Sal3 and Sal4, two different culture collection strains of *S. salivarius*, respectively; Mu1 and Mu2, two colonies of *S. mutans* DSM 20523, respectively; Mu3 and Mu4, two different culture collection strains of *S. mutans*, respectively.

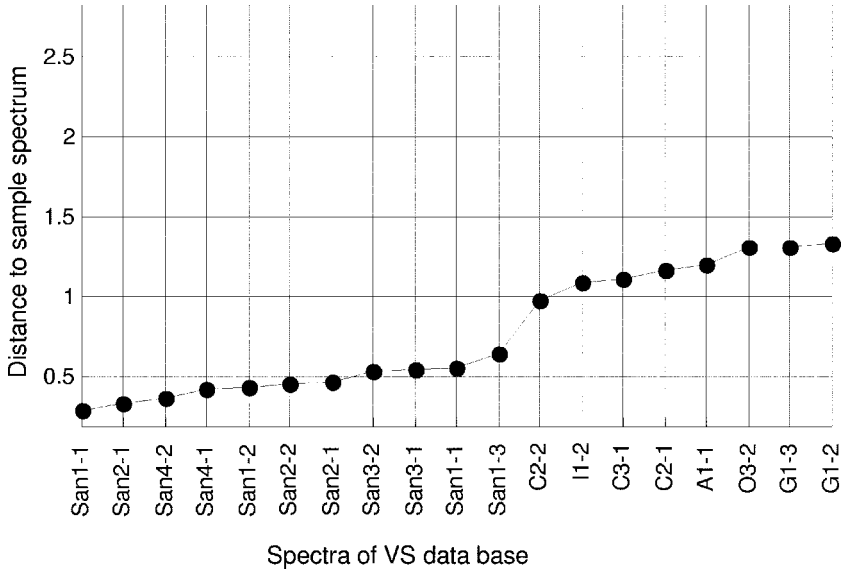

FIG. 3. Results of the identification of one clinical sample (isolate VS 162) on the basis of a similarity analysis of the MALDI-TOF mass spectra. The peak list generated from the spectrum of VS 162 was compared to the peak lists of all reference and culture collection strains (VS database). On the *y* axis, the distance between the spectrum of VS 162 and the spectra of the references and culture collection (all measured in triplicate) is given. On the *x* axis, the entries of the VS database are arranged according to the increasing dissimilarities of their MALDI-TOF-MS spectra compared to the spectrum of VS 162. For clarity, only the results for the 19 most similar entries of the VS database are shown. San1-1, San1-2, and San1-3, triplicate cultures of *S. sanguinis* DSM 20567; San2-1, San2-2, San3-1, San3-2, San4-1, and San4-2, duplicate cultures of three different culture collection strains of *S. sanguinis*; G1-2 and G1-3, duplicate cultures of *S. gordonii* DSM 6777 Il-2, *S. intermedius* DSM 20573; C2-1, C2-2, and C3-1, duplicate cultures and single culture of two different culture collection strains of *S. constellatus*; A1-1, culture collection strain of *S. anginosus*; O3-2, culture collection strain of *S. oralis.* Identical entries at different positions on the *x* axis represent duplicate MALDI-TOF mass spectra from one culture.

given in Fig. 4a) or *S. mitis* (an example is given in Fig. 4b). For only two isolates (isolates VS 23 and VS 157) the results were not clear (an example is shown in Fig. 4c). Therefore, an alternative method for the differentiation of *S. oralis* and *S. mitis* was developed. It rests on the classification of the spectra by use of an SVM algorithm. SVM algorithms are a class of statistical learning algorithms whose theoretical basis was first presented by Vapnik (30). It has been successfully applied to numerous classification and pattern recognition problems in bioinformatics (12). A database (mitis/oralis database) which consisted of the MALDI-TOF-MS spectra of the *S. mitis* and *S. oralis* reference and culture collection strains, together with those of previously identified clinical isolates of the two species, was constructed. This database was cross-validated by using the SVM algorithm. The sensitivity, specificity, and correct rate were all found to be 1.0. By classification by use of the SVM algorithm, isolates VS 23 and VS 157 were both unambiguously identified as *S. mitis*.

TABLE 1. Identification of 99 VS strains by MALDI-TOF MS and reference methods

VS	No. of isolates identified by[a]:	
	MALDI-TOF-MS	Reference methods
Mitis group	71	71
S. oralis	37	34
S. mitis	23	3
S. mitis/*S. oralis*	0	23
S. parasanguinis	10	10
S. gordonii	0	0
S. sanguinis	1	1
Salivarius group	5	5
S. salivarius	5	5
Anginosus group	23	23
S. anginosus	19	19
S. intermedius	2	2
S. constellatus	2	2
Mutans group	0	0
S. mutans	0	0

[a] A total of 99 isolates were tested by each method. The reference methods are the test with the API system, species-specific PCR, and 16S rRNA gene sequencing.

For the past three decades, a number of mass spectrometry-based methods have been developed for the rapid differentiation of bacteria phenotypically. Ionization of bacterial extracts by fast-atom bombardment, electrospray ionization, and sample pyrolysis, followed by MS and MS/MS, have been reported (2, 6, 20, 31). More recently, whole-cell MALDI-TOF-MS has been used to quickly differentiate microorganisms (19). Based on abundant peptides and small proteins, MALDI-TOF-MS generates complex spectra which contain unique *m*/*z* signatures for different microorganisms due to the inherent variations in their masses and/or the abundance of the related proteins that they express. The MALDI-TOF-MS spectra can be analyzed with respect to the detection of one or several specific peaks (15, 22, 26, 32) or by analyzing the complete spectral pattern (4).

In conclusion, our findings indicate that MALDI-TOF-MS, in combination with pattern analysis, may be useful for

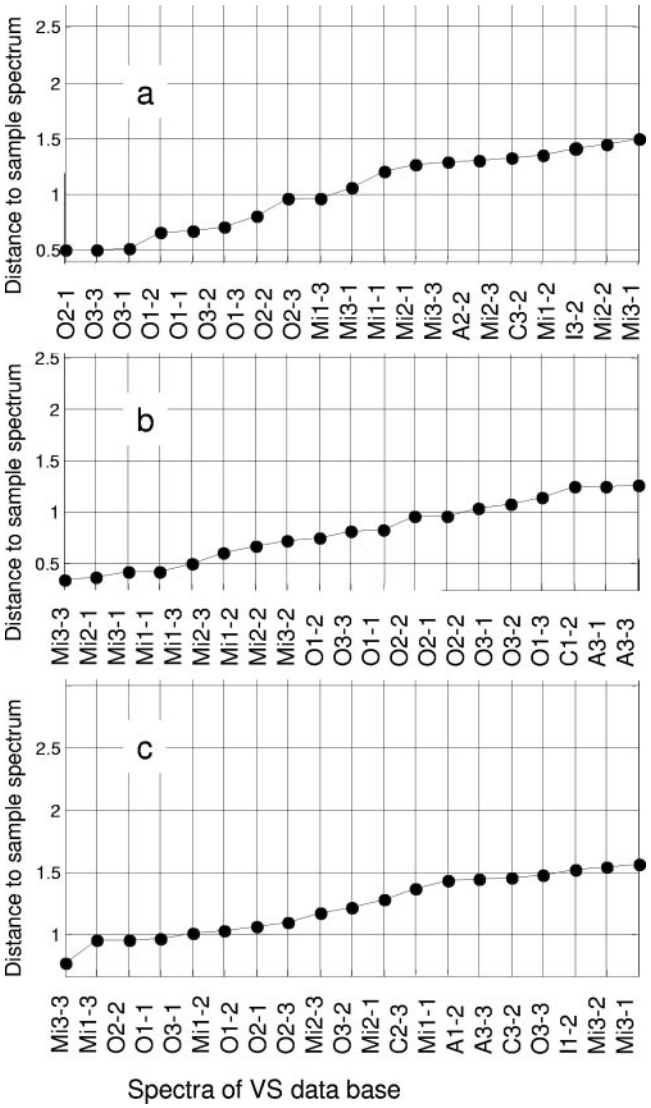

FIG. 4. Examples of results of the identification of the 23 samples which could be identified as either *S. mitis* or *S. oralis* by species-specific PCR and sequence analysis of the 16S RNA gene (Table 1, *S. oralis*/*S. mitis* [one clinical sample]) on the basis of a similarity analysis of the MALDI-TOF mass spectra. The peak list generated from the spectra were compared to the peak lists of all reference and culture collection strains (VS database). On the *y* axis, the distance between the spectrum of the respective clinical isolate and the spectra of the reference and culture collection isolates (all measured in triplicate) is given. On the *x* axis, the entries of the VS database are arranged according to the increasing dissimilarities of their MALDI-TOF-MS spectrum compared to the spectrum of the respective clinical isolate. (a) Results of identification of a clinical sample which can be identified as *S. oralis*; (b) results of identification of a clinical sample which can be identified as *S. mitis*; (c) results of identification of a clinical sample (VS 23) which cannot be identified as either *S. oralis* or *S. mitis*. See the legends to Fig. 2 and 3 for an explanation of the VS database designations.

the rapid and accurate identification of VS. Currently, however, the high investments necessary for this methodology might preclude routine microbiological laboratories from using this technology. Established genotyping methods based on the 16S rRNA gene do not allow the correct differentiation of *S. oralis* and *S. mitis* because the intraspecies sequence variations may be higher than the interspecies variations (7, 23, 28). In contrast, phenotypic classification with the SVN algorithm based on MALDI-TOF mass spectra allowed the accurate differentiation between *S. oralis* and *S. mitis*. Discrimination between *S. oralis* and *S. mitis* is of clinical relevance, since some studies have shown that *S. mitis* strains are more resistant to penicillin, fluoroquinolones, and three or more classes of antibiotics (13, 24).

ACKNOWLEDGMENTS

We thank A. Hennig-Rolle, H. Stache, and H. Boitz for technical assistance.

The work was supported by an award of the Virtual Institute from the Impuls- und Vernetzungsfond of the Helmholtz Association.

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